resistant isolates. In addition, β-17 is non-hemolytic, an indication that this molecule has retained the inherent selectivity of the natural constructs.

### Concluding remarks

There are several reasons why one must take notice of Gellman's achievement. β-17 is a very different molecule from one composed of natural amino acids, and provides us with a different fundamental design to build upon as we go forward in the creation of therapeutics. Because of unpredictable toxicologies that surround a class of compound as it is being developed, a new chemical class provides freedom to continue to exploit a therapeutic target but not be limited to a specific chemical design. The simplicity of the molecule might help further elucidate differences in membrane design between animal cells and microbes that underlie specificity, and as such permit further refinement of antimicrobial peptide design. The benefit of a molecule that is protease resistant is evident as one considers applications where peptide degradation is of concern, as in intravenous therapy. What will ultimately establish the utility of this class of compounds as a therapy will reduce down to, safety, pharmacological properties in humans, the cost of synthesis, and their efficacy as therapeutic agents.

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# How accurately can we image inositol lipids in living cells?


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There are few cellular processes in which inositol phospholipids have not been reported to play a regulatory role. In the 1980s, the unfolding biochemical details of the receptor-activated hydrolysis of phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)$P_2$] by phospholipase C (PLC) enzymes, revealed the concerted roles of the $Ca^{2+}$-mobilizing messenger inositol (1,4,5)-trisphosphate [Ins(1,4,5)$P_3$], and the protein kinase C (PKC) activator diacylglycerol in cellular signalling. In addition to this precursor function, more recently, inositol phospholipids *per se* have been identified as dynamic regulators of the assembly of several signalling complexes in localized membrane compartments. The local concentration of inositol lipids (which differ in the number and position of phosphate groups around their inositol ring) is determined by the targeting and activation mechanism(s) of the numerous enzymes that are responsible for their production and degradation. Once formed, these membrane-bound messengers recruit additional signalling molecule(s) that possess molecular determinants for the recognition of individual inositol phospholipids. Studies on the compartmentalized regulatory role of inositides require methods that allow detection of lipid distribution and dynamics with spatial and temporal resolution, as opposed to the conventional means by which changes in the amounts and/or the turnover of overall cellular inositol lipid pools are determined.

### Use of protein modules that recognize inositol lipids to visualize lipid distribution

The functions of an increasing number of signalling proteins (e.g. ion channels, ion pumps, protein and lipid kinases and phosphatases, and regulators of

**Table 1. Visualization of phosphoinositides by protein-domain–GFP chimeras in live cells**

| Lipid | Protein domain | Cellular localization[a] | Refs |
|---|---|---|---|
| PtdIns(4,5)$P_2$ | PLC$\delta_1$-PH | Plasma membrane | 5,14 |
| PtdIns(4,5)$P_2$ or PtdIns(4)$P$ | OSBP-PH | Golgi membrane | 3 |
| PtdIns(3,4,5)$P_3$ | ARNO-PH | Plasma membrane[b] | 15,16 |
| | BTK-PH | Plasma membrane[b] | 17 |
| | GRP1-PH | Plasma membrane[b] | 16,18 |
| | Cytohesin-1-PH | Plasma membrane[b] | 12,19 |
| PtdIns(3,4,5)$P_3$ or PtdIns(3,4)$P_2$ | Akt-PH | Plasma membrane[b] | 18,20 |
| PtdIns(3)$P$ | EEA1-FYVE | Endocytic vesicle | 6 |

[a]The lack of localization to certain membranes does not rule out the presence of the lipid in those compartments.
[b]Only after activation of phosphatidylinositol 3-kinases.
Abbreviations: Akt, protein kinase B; ARNO, Arf nucleotide opener; BTK, Bruton's tyrosine kinase; EEA1, early-endosome antigen; GFP, green fluorescent protein; GRP1, general receptor of phosphoinositides 1; OSBP, oxysterol-binding protein; PH, pleckstrin homology; PLC, phospholipase C; PtdIns(3)$P$, phosphatidylinositol (3)-phosphate; PtdIns(3,4)$P_2$, phosphatidylinositol (3,4)-bisphosphate; PtdIns(4)$P$, phosphatidylinositol (4)-phosphate; PtdIns(4,5)$P_2$, phosphatidylinositol (4,5)-bisphosphate; PtdIns(3,4,5)$P_3$, phosphatidylinositol (3,4,5)-trisphosphate.


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either GTP-binding proteins or the cytoskeleton) have been shown to be regulated, with remarkable stereospecificity, by inositol lipids. In many of these cases the protein motifs that mediate the interactions with the various inositol lipids have been defined, often with detailed three-dimensional structural information. These domains possess characteristic protein folds such as pleckstrin homology (PH) domains, FYVE domains, and the C1 and C2 domains (cysteine-rich regions) present in several signalling proteins[1], and retain their lipid recognition properties even when expressed separately without the rest of their parent molecule.

These recent advances raised the possibility that minimum lipid-recognition sequences fused to green fluorescent protein (GFP) could represent novel tools to identify inositol lipids at their cellular sites of production, and permit their dynamic imaging in living cells under a confocal microscope. Indeed, several recent studies have used such protein domains fused to GFP to demonstrate their specific interaction with membrane inositides in live cells (Table 1 and Fig. 1). This technique represented a major step in the advancement of the technology for studying inositol (and other) lipids, and also provided the possibility to determine the lipid-specificity of these protein modules in their natural membrane environment. It should be noted, however, that the bulky GFP molecule could alter the behaviour of the attached protein module, although it has been found to be remarkably 'inert' in many applications.

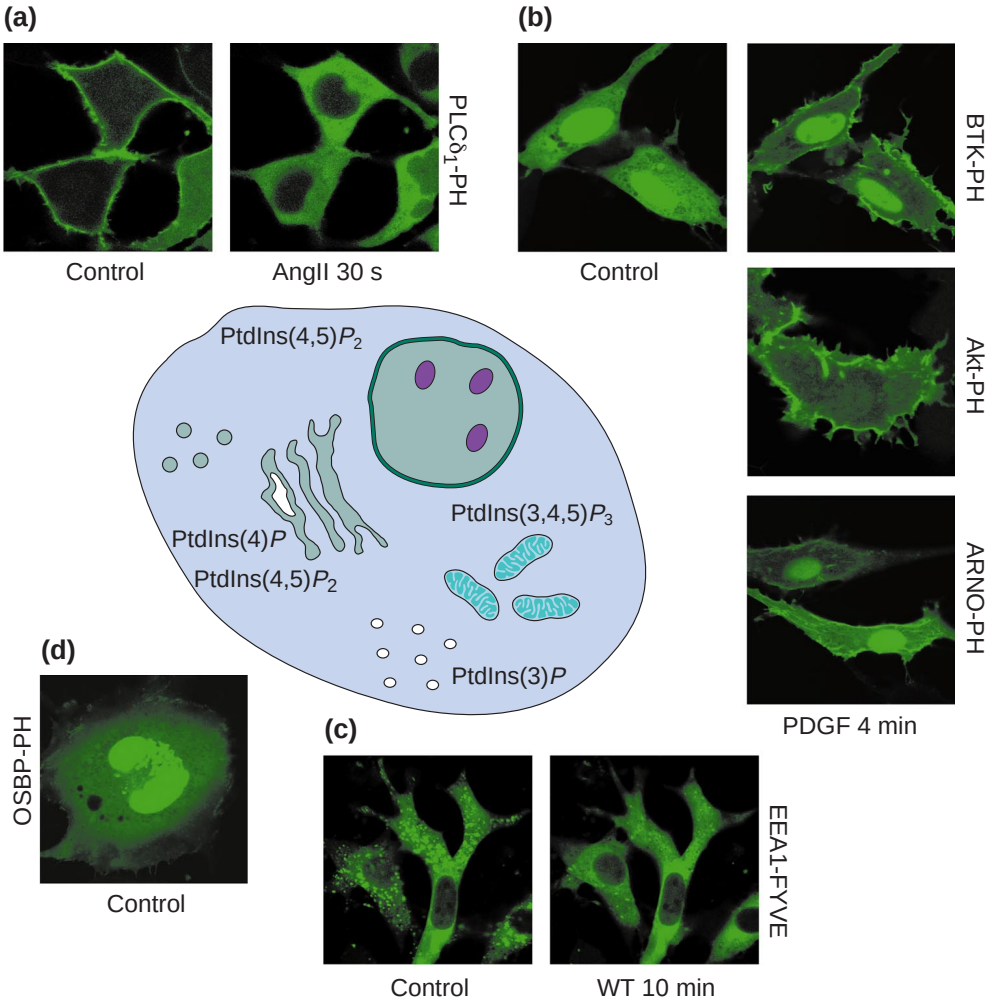


**Fig. 1.** The cellular distribution of protein modules fused to green fluorescent protein (GFP) is shown. **(a)** Phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)$P_2$] of the plasma membrane (but not of internal membranes) is identified by the localization of the chimera between the pleckstrin homology (PH) domain of phospholipase C $\delta_1$ (PLC$\delta_1$) and GFP (control). The rapid breakdown of this inositol lipid after stimulation with angiotensin II (AngII) is reflected in the translocation of the fluorescent chimera to the cytosol. **(b)** By contrast, the probes that detect phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)$P_3$] (i.e. Akt-PH–GFP, BTK-PH–GFP and ARNO-PH–GFP chimeras) are cytosolic in quiescent cells (control) and translocate to the plasma membrane only after stimulation by agonists [e.g. platelet-derived growth factor (PDGF)] that promote production of the lipid. As a result of the presence of several basic residues and their relatively small size, some of these probes also accumulate in the nucleus. **(c)** Localization of the chimera between the FYVE domain of the early endosomal autoantigen (EEA1) protein and GFP shows the constitutive presence of phosphatidylinositol (3)-phosphate [PtdIns(3)*P*] on the membranes of endocytic vesicles (control), which slowly disappears when lipid production is inhibited by the phosphatidylinositol 3-kinase inhibitor, wortmannin (WT). **(d)** The Golgi localization of the PH domain of oxysterol-binding protein (OSBP) tagged with GFP reflects its binding to phosphatidylinositol (4)-phosphate [PtdIns(4)*P*] or PtdIns(4,5)$P_2$ together with a Golgi-specific factor[3]. It should be noted that the lack of localization to certain membranes does not rule out the presence of the lipid in those compartments. Abbreviations: Akt, protein kinase B; ARNO, Arf nucleotide opener; BTK, Bruton's tyrosine kinase.

### Can all the cellular lipids be 'imaged' with these probes?

Although these studies convincingly demonstrate that the distribution of these fluorescent probes is dependent on the presence or production of a particular inositol lipid, the question has been raised, from the beginning, whether the method allows equal detection of all of the cellular inositides. For example, tagging of the PH domain of PLC$\delta_1$ with GFP recognizes plasma membrane PtdIns(4,5)$P_2$ but fails to label intracellular structures (e.g. Golgi, the nuclear envelope or secretory vesicles) where the presence of PtdIns(4,5)$P_2$ has been established biochemically[2]. By contrast, GFP bound to the PH domain of the oxysterol-binding protein (OSBP), which has also been shown to bind PtdIns(4,5)$P_2$, localizes primarily to Golgi membranes, with only a low level of signal detectable at the plasma membrane[3]. The lack of localization of PLC$\delta_1$-PH–GFP to secretory vesicles[4] is of particular interest because the need for PtdIns(4,5)$P_2$ synthesis in the 'priming' of the secretory machinery has been firmly established[2] and the presence of PtdIns(4,5)$P_2$ can be detected on the surface of some secretory vesicles in permeabilized PC-12 cells by antibodies raised against PtdIns(4,5)$P_2$ (T.F. Martin, pers. commun.). This could indicate that this lipid pool binds other proteins and, therefore, is not available for binding PLC$\delta_1$-PH–GFP, but still could be detected by an antibody after permeabilization.

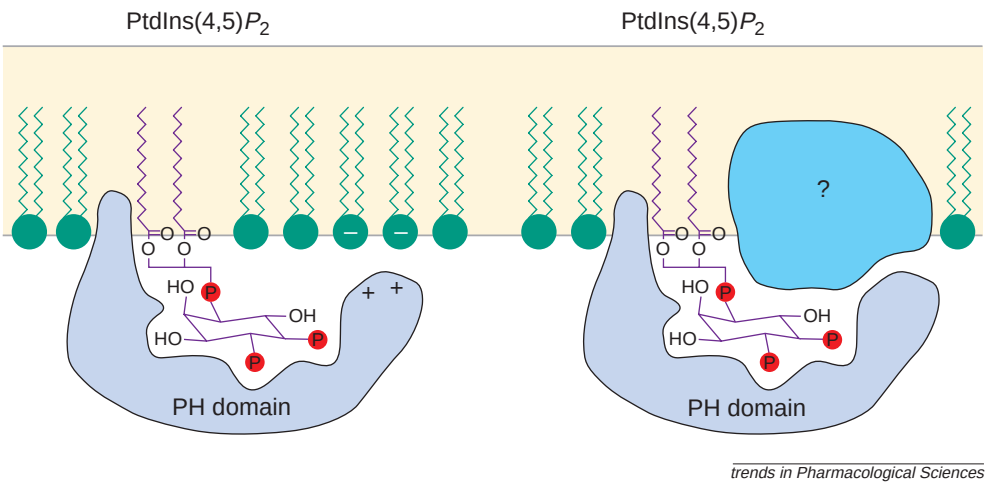


**Fig. 2.** Anchoring of isolated pleckstrin homology (PH) (or other lipid-binding) domains to cellular membranes by inositide-specific interactions. In addition to the specific recognition of the inositol lipid head group, hydrophobic residues that penetrate the membrane, as well as additional electrostatic interactions with other acidic phospholipids might contribute to membrane localization (left). Possible auxiliary interactions with membrane-specific proteins could restrict recruitment of inositide-binding domains into specific membrane compartments (right). In this latter model the modules serve as coincidence detectors allowing more specific control over simultaneously occurring signalling events.

Additionally, there is a discrepancy between the kinetics of changes in *myo*-[$^3$H]inositol-labelled PtdIns(4,5)$P_2$ pools and those that bind PLC$\delta_1$-PH–GFP. For example, the resynthesis of labelled PtdIns(4,5)$P_2$ pools is almost completely inhibited by micromolar concentrations of wortmannin [either after agonist stimulation or after an ionomycin-induced PtdIns(4,5)$P_2$ hydrolysis], whereas the translocation responses of PLC$\delta_1$-PH–GFP indicate no obvious wortmannin sensitivity of the synthesis of the 'imaged' PtdIns(4,5)$P_2$ pools[5].

There is lesser ambiguity in the recognition of PtdIns(3,4,5)$P_3$ by the PH domains of BTK (Bruton's tyrosine kinase), Akt (protein kinase B), or the group of ARNO-family proteins, but this might only be because 3-phosphorylated lipids have not been so thoroughly characterized with conventional methods. However, it is notable that none of the PH domains that has been used to bind PtdIns(3,4,5)$P_3$ detected this lipid in intracellular membranes. By contrast, based on the distribution of the early endosomal autoantigen (EEA1)-FYVE-domain–GFP chimera, PtdIns(3)$P$ would only be formed on the surface of early endosomes[6]. Although it is possible that PtdIns(3)$P$ is present only in those membranes, it is more likely that the EEA1-FYVE-domain–GFP construct can recognize PtdIns(3)$P$ only in certain molecular contexts, namely together with the activated GTP-binding protein, Rab5 (Ref. 7).

### Changes in the levels of soluble inositol phosphates can complicate interpretations

A further issue to be considered when interpreting data on the cellular distribution and dynamics of the PH domains[8] is the equal or higher apparent *in vitro* affinity of these proteins to the soluble inositol phosphate isomers compared with the cognate inositol lipid. A good example is again PLC$\delta_1$-PH, which binds Ins(1,4,5)$P_3$ with about 10 times higher affinity than PtdIns(4,5)$P_2$ *in vitro*. Based on this finding, it has been proposed that translocation of the PLC$\delta_1$-PH–GFP protein from the membrane to the cytosol is a reflection of the cytosolic increase in Ins(1,4,5)$P_3$ rather than the decreased PI(4,5)$P_2$ content of the membrane[9]. An analogous situation exists for the PtdIns(3,4,5)$P_3$-recognizing PH domains, where their Ins(1,3,4,5)$P_4$ binding complicates interpretations on their localization to, or release from, the plasma membrane[10]. More experiments are needed to determine the extent to which these factors affect the distribution of the probes.

### Additional interactions might complement lipid-binding and determine membrane localization of the fluorescent probes

The interpretation of data obtained by following the distribution of these probes depends on whether the binding to the inositol lipid head group is the sole determinant of their membrane association. A protein might not necessarily localize to the plasma membrane simply because it binds Ins(1,4,5)$P_3$ [or the inositol lipid head group of PtdIns(4,5)$P_2$]. For example, the isolated Ins(1,4,5)$P_3$-binding domain of the Ins(1,4,5)$P_3$ receptor does not localize to the plasma membrane (P. Várnai and T. Balla, unpublished). Hydrophobic residues that allow penetration of the domain to the hydrophobic membrane, and/or additional interactions with acidic phospholipids such as phosphatidylserine, might be necessary for membrane recruitment. However, if the additional interactions are provided by specific membrane proteins, the lipid-dependent localization of the domain will also be determined by the distribution of the auxiliary protein-binding partner (Fig. 2). This could also explain why lipids in certain membranes are not detected by the fluorescent probes. In fact, several reports have revealed binding of these motifs to various other proteins, although these are not dependent on lipids *in vitro*. Examples include the binding of the BTK-PH domain to βγ-subunits of heterotrimeric G proteins or to C1 domains of various PKC isoforms[11]. These protein–protein interactions do not explain the lipid-dependent translocation responses of the lipid-binding domains in question but might suggest a more complex recognition mechanism that determines their recruitment to cellular membranes. This idea is supported by the observation that no PtdIns(3,4,5)$P_3$-dependent translocation response of the cytohesin-1 PH domain is observed unless the polybasic sequence adjacent to the C-terminus of the PH domain is included in the PH-domain–GFP construct[12]. This basic stretch is not believed to contribute to the PtdIns(3,4,5)$P_3$ binding pocket. Similarly, a minimum PtdIns(3)$P$ recognition sequence within the FYVE domain of the EEA1 protein,

does not show PtdIns(3)*P*-dependent membrane localization without the N-terminally adjacent α-helix[6,13], which is probably part of the Rab5-binding domain[7] or could participate in dimerization. Until these questions are critically evaluated, caution is advisable when making conclusions about the lack of lipids in certain membrane compartments based on localization studies with such protein constructs.

Another important question is how the expression of the protein module can distort lipid distribution. This is especially relevant if the 'minimal' domain can interact with membrane proteins, because it will sequester a sizeable portion of the inositol lipid pool to the compartment where the protein binding partner resides. Consequently, by taking a 'look' into the cell we can significantly change the molecular geography that we are trying to discover. Increased lipid affinity of the modules and better fluorescence quantum efficiency are keys to overcome such distortions. In addition, one also has to consider that over-expression of inositide-binding protein probes might exert an inhibitory effect on various lipid-dependent cellular functions. This could result in negative selection and enrichment of cells that possess adaptive mechanisms to correct for such loss of function, when one tries to create stable transfectants with these domains.

### Concluding remarks

The reader might be wondering whether this recently developed elegant and promising technique provides more trouble than benefit, but this is certainly not the case. In fact, we strongly believe that the use of these novel tools will permit the exploration of the inositide-regulatory paradigm at a different level and, more importantly, from a new direction. Based on these studies we have realized that not all inositol lipids exist in a 'free' form and some must be complexed with proteins. Dominant-negative effects of the constructs can be used to dissect and analyse lipid-mediated signalling steps. If the lipid-dependent distribution of a particular probe is proven to be restricted by other factors (e.g. protein), one could follow inositide changes that are functionally coupled to a specific signalling pathway. The desire to image lipids and critically evaluate the limitations of this approach is only an additional catalyst for our thinking to better understand the principles that govern inositide-based control of such numerous and diverse cellular functions.

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